Selective labelling and inactivation of creatine kinase isoenzymes by the thyroid hormone derivative *N*-bromoacetyl-3,3′,5-tri-iodo-L-thyronine

Markus WYSS,* Theo WALLIMANN*‡ and Josef KÖHRLE†§

*Swiss Federal Institute of Technology, Institute for Cell Biology, ETH-Hönggerberg, CH-8093 Zürich, Switzerland, and †Abteilung Klinische Endokrinologie, Medizinische Hochschule, D-3000 Hannover 61, Germany

Besides their well-known regulation of transcription by binding to nuclear receptors, thyroid hormones have been suggested to have direct effects on mitochondria. In a previous study, incubation of rat heart mitochondria with ^{125}I-labelled *N*-bromoacetyl-3,3′,5-tri-iodo-L-thyronine ($BrAcT_3$), a thyroid hormone derivative with an alkylating side chain, resulted in the selective labelling of a protein doublet around M_r 45000 on SDS/polyacrylamide gels [Rasmussen, Köhrle, Rokos and Hesch (1989) FEBS Lett. **255**, 385–390]. Now, this protein doublet has been identified as mitochondrial creatine kinase (Mi-CK). Immunoblotting experiments with the cytoplasmic and mitochondrial fractions of rat heart, brain and liver, as well as inactivation studies with the purified chicken CK isoenzymes have further demonstrated that all four CK isoenzymes (Mi_a-, Mi_b-, M- and B-CK) are indeed selectively labelled by $BrAcT_3$. However, in contrast with their bromoalkyl derivatives, thyroid hormones themselves did not compete for CK labelling, suggesting that not the thyroid hormone moiety but rather the bromoacetyl-driven alkylation of the highly reactive 'essential' thiol group of CK accounts for this selective labelling. Therefore the assumption that CK isoenzymes are thyroid-hormone-binding proteins has to be dismissed. Instead, bromoacetyl-based reagents may allow a very specific covalent modification and inactivation of CK isoenzymes *in vitro* and *in vivo*.

INTRODUCTION

Creatine kinase (CK, EC 2.7.3.2) isoenzymes are involved in the energy metabolism of tissues with high and fluctuating energy demands such as skeletal and cardiac muscle, brain, photoreceptor cells of the retina and spermatozoa (for reviews see [1,2]). In chicken, rat and man, four different CK isoenzymes are known, two cytoplasmic (M- and B-CK) and two mitochondrial (Mi_a- and Mi_b-CK), all with a subunit M_r of approx. 43000. M- and Mi_b-CK are found primarily in sarcomeric muscles, whereas B- and Mi_a-CK are the predominant CK isoenzymes in neural tissues.

At the cellular level, two different modes of direct action of thyroid hormones have been put forward. The nuclear effects are well established and are mediated by the binding of triiodothyronine (T_3) to specific nuclear c-*erbA*-related receptor proteins with subsequent transcriptional activation or inhibition of T_3-responsive genes (for a review see [3]). On the other hand, immediate stimulation of ion, sugar and amino acid transport across the plasma membrane, increased oxygen consumption in mitochondria and alterations of the filamentous cytoskeleton were proposed as extranuclear effects of thyroid hormones, occurring independently of nuclear regulation of transcription by T_3 ([4,5]; for reviews see [6–8]). However, the occurrence and physiological significance of these latter effects is still disputed.

In order to identify mitochondrial thyroid-hormone-binding proteins, Rasmussen et al. [9] made use of ^{125}I-labelled *N*-bromoacetyl-T_3 ($BrAc[^{125}I]T_3$). This alkylating T_3 derivative and its thyroxine analogue ($BrAcT_4$) have been extensively used for the identification of thyroid-hormone-binding proteins of the nucleus, the nuclear envelope, the endoplasmic reticulum and the plasma membrane ([10–20]; for further references see [21]). As a matter of fact, incubation of rat heart mitochondria with $BrAc[^{125}I]T_3$ resulted in the very selective labelling of a protein doublet with an apparent M_r of about 45000 [9].

Several arguments made it attractive to suggest that this protein doublet represents Mi-CK and that interaction of thyroid hormones with Mi-CK is of physiological importance. (1) The $BrAcT_3$-labelled protein doublet and purified rat heart Mi-CK have approximately the same M_r [9,22]. Furthermore, under certain conditions, rat heart Mi-CK also migrates as a double band in SDS/polyacrylamide gels [22]. (2) Mitochondrial T_3-binding proteins were previously localized to the inner membrane ([23,24]; for a review see [7]) and coincide therefore in location with Mi-CK which is attached to the outer surface of the inner mitochondrial membrane [25]. (3) Thyroid hormones influence and regulate the energy metabolism of cells. On the other hand, Mi-CK fulfils all preconditions to be an attractive site for regulation of cellular energy metabolism. In contrast with their cytosolic counterparts, Mi-CK isoenzymes are thought to be displaced from thermodynamic equilibrium and thus are under kinetic control [26,27]. Regulation of the enzyme activity of Mi-CK by thyroid hormones could thus directly influence the flux of high-energy phosphates out of the mitochondria. (4) Similarly to presumptive thyroid-hormone-binding proteins such as thyroid peroxidase [28], thyroxine-binding globulin [29], 5- and 5′-iodothyronine monodeiodinases [18,19,30], protein disulphide isomerase [16], and the nuclear T_3 receptor [31], all CK iso-

Abbreviations used: CK, creatine kinase; M-, B-, and Mi-CK, subunit isoforms of muscle-type, brain-type and mitochondrial creatine kinase; Mi_a-CK, brain isoform of mitochondrial creatine kinase; Mi_b-CK, heart isoform of mitochondrial creatine kinase; PCr, phosphocreatine; 3,5-T_2, 3,5-di-iodo-L-thyronine; T_4, L-thyroxine; $BrAcT_3$, *N*-bromoacetyl derivative of T_3; $BrAc[^{125}I]T_3$, ^{125}I-labelled $BrAcT_3$; $NAcT_3$, *N*-acetyl-T_3; $NAcT_4$, *N*-acetyl-T_4; ANT, adenine nucleotide translocator; 2-ME, 2-mercaptoethanol; DNFB, 2,4-dinitrofluorobenzene; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); BAEE, N_α-benzoyl-L-arginine ethyl ester.

‡ To whom correspondence and reprint requests should be addressed.

§ Present address: Medizinische Poliklinik der Universität Würzburg, Klinische Forschergruppe, Klinikstr. 6-8, D-8700 Würzburg, Germany.

enzymes also contain in or near the active site a highly reactive thiol group, alkylation of which leads to almost complete inactivation of the enzyme (for a review see [32]). This might suggest that a reactive thiol group is an essential prerequisite for thyroid hormone binding. (5) Already in the 1950s, it was reported that thyroxine influences CK activity (for a review see [33]), but significant effects were only observed at unphysiologically high T_4 concentrations.

The aims of the present investigation were (1) to identify the protein doublet labelled by $BrAcT_3$ in rat heart mitochondria, (2) to extend these studies to the cytosolic and mitochondrial fractions of rat brain and liver in order to see if labelling by $BrAcT_3$ is a particular property of rat heart Mi_b-CK or a more general phenomenon of all known CK isoenzymes and (3) to characterize the nature of the interaction of $BrAcT_3$ with Mi_b-CK. The present findings may serve as a basis for the design of highly specific labelling and inactivation reagents for all CK isoenzymes.

MATERIALS AND METHODS

Materials

H.p.l.c.-purified thyroid hormones, thyroid hormone derivatives and iopanoic acid were kindly provided by Dr. H. Rokos (Henning GmbH, Berlin, Germany). EMD 21388, a T_4-antagonistic flavonoid [19], was obtained from Dr. K. Irmscher (E. Merck, Darmstadt, Germany). $BrAcT_3$, $BrAcT_4$ and $BrAc[^{125}I]T_3$ (specific radioactivity 3 mCi/μg) were synthesized as described [18]. The disodium salts of ADP and ATP as well as papain were purchased from Boehringer-Mannheim (Germany), creatine was from Sigma (St. Louis, MO, U.S.A.), phosphocreatine (PCr) from Calbiochem (Lucerne, Switzerland), bromoacetic acid, phenacyl bromide, methyl bromoacetate, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and N_α-benzoyl-L-arginine ethyl ester (BAEE) were from Fluka (Buchs, Switzerland), and 2-bromoacetamide and 2-(2-bromoacetyl)naphthalene from Aldrich (Suchema AG, Kaltenbach, Switzerland). All other chemicals were at least of reagent grade. Chicken Mi_a-CK (specific activity 62.5 units/mg of protein), Mi_b-CK (115 units/mg of protein) and B-CK (230 units/mg of protein) were purified as previously described [34–36]. Chicken M-CK (210 units/mg of protein) was purified as described by Eppenberger et al. [37] followed by anion-exchange chromatography on a Mono Q column (f.p.l.c.; Pharmacia, Dübendorf, Switzerland).

Preparation of mitochondria, mitoplasts and cytosolic fractions from rat heart, brain and liver

All steps of the preparation were performed at 4 °C or on ice, and all homogenizations and resuspensions were done with a tightly fitting glass/Teflon homogenizer at 400 rev./min. After homogenization of two rat hearts (or two brains or two livers) in 60 ml (45 ml or 60 ml) of buffer A [220 mM mannitol, 70 mM sucrose, 10 mM Hepes, 0.2 mM EGTA, 1 mM 2-mercaptoethanol (2-ME), 1 mM NaN_3, pH 7.4] and centrifugation at 750 *g* for 5 min to remove whole cells, nuclei and fibrillar material, mitochondria were pelleted and washed by two consecutive centrifugations at 6000 *g* for 10 min, followed by resuspension in 20 ml (25 ml or 50 ml) of buffer A. After a further centrifugation at 6000 *g* for 15 min, heart and liver pellets were resuspended in 5 ml and 30 ml of buffer A respectively. The fractions thus obtained represent purified whole mitochondria. The brain pellet was swollen in 30 ml of bidistilled water to open synaptic vesicles and therefore remove B-CK. The mitoplasts were pelleted by two consecutive centrifugations at 10000 *g* and 20000 *g* for 30 min, followed by resuspension in 25 ml of bidistilled water and 10 ml of buffer A respectively. The final fraction contains purified brain mitoplasts. The supernatants of the very first centrifugation at 6000 *g* were used as heart, brain and liver cytosolic fractions.

Labelling experiments

Mitochondrial, mitoplast and cytosolic fractions of rat heart, brain and liver at a protein concentration of 1 mg/ml as well as purified chicken Mi_a- and Mi_b-CK at a subunit concentration of 200 nM (8.6 μg/ml) (100 μl of each) were preincubated in buffer A for 10 min at 25 °C in the presence of the respective substrates or competitors. Five-fold-concentrated stock solutions of substrates in buffer A and 50-fold-concentrated stock solutions of thyroid hormones and thyroid hormone derivatives in methanol were used throughout the experiments. Then, 60–100 nCi of $BrAc[^{125}I]T_3$ (specific radioactivity 3 mCi/μg) in 2 μl of methanol was added and incubations were continued at 25 °C for the indicated periods of time. The reactions were stopped by the addition of 35 μl of 4-fold-concentrated SDS/PAGE sample buffer (final concentrations: 2% lithium dodecyl sulphate, 51 mM thioglycollic acid, 100 mM Tris/HCl, pH 6.8, 7.5% glycerol). Then 20 μl of each of these samples was transferred to 200 μl of ice-cold ethanol. After a 10 min incubation on ice, proteins and therefore also protein-bound radioactivity were pelleted at 15000 *g* for 10 min in a microcentrifuge. Supernatants were removed, and the radioactivities in pellets and supernatants determined in a γ-counter. The remaining 115 μl of the samples were boiled for 5 min at 95 °C and then directly used for SDS/PAGE.

Control experiments demonstrated that the rate of labelling by $BrAc[^{125}I]T_3$ of purified chicken Mi_a- and Mi_b-CK, rat heart mitochondria and rat brain mitoplasts was almost linear over at least 30 min. In the other fractions, the rate of labelling significantly decreased after 10–15 min, but the pattern of labelled bands on SDS/polyacrylamide gels did not change for incubation times up to 30 min.

Inactivation of Mi_b-CK with bromoacyl compounds

Purified CK isoenzymes were extensively dialysed at 4 °C against buffer B (50 mM sodium phosphate, 150 mM NaCl, 0.2 mM EDTA, 0.5 mM 2-ME, 2 mM NaN_3, pH 7.2). Inactivation experiments with $BrAcT_3$, bromoacetic acid, 2-bromoacetamide, methyl bromoacetate, phenacyl bromide or 2-(2-bromoacetyl)-naphthalene were performed in buffer B at a CK subunit concentration of 4 μM (0.17 mg/ml) and at 25 °C for the indicated periods of time. For competition experiments, chicken Mi_b-CK was preincubated for 10 min at 25 °C in the presence of the respective substrates or competitors. Substrates were added as stock solutions in buffer B, whereas 40-fold-concentrated stock solutions of all bromoacyl compounds and 10-fold-concentrated stock solutions of thyroid hormones in methanol were used. In control experiments, methanol at the concentrations employed had no effect on CK activity. CK activity measurements were performed by the pH-Stat method in the direction of ATP synthesis in an assay mixture containing 65 mM KCl, 8.5 mM $MgCl_2$, 85 μM EGTA, 4 mM ADP and 10 mM PCr. As titrant, 20 mM HCl was used. One unit corresponds to 1 μmol of PCr transphosphorylated/min at 25 °C and pH 7.00. For the graphical representation of the data, semilogarithmic plots were chosen (Figures 3 and 5) in order to visualize more clearly the

differences between the distinct sets of conditions. Initial inactivation rate constants were calculated as follows. In an irreversible reaction $E + R \rightarrow M$ with rate constant k, where E signifies the unmodified CK isoenzyme, R the respective reagent, and M the modified inactivated CK isoenzyme, the equation:

$$kt = \frac{\ln(e_o r / r_o e)}{r_o - e_o}$$

(e_o, r_o, concentrations of E and R at time $t = 0$; e, r, concentrations of E and R at any given time t) can be applied for the calculation of k. The initial inactivation rate constants were calculated from the 0 and 10 min values shown in the Figures.

The feasibility of this approach was checked by performing inactivation experiments of Mi_b-CK with 1, 2.5, 5, 10, 20 and 40 μM $BrAcT_3$. The inactivation rate proved to depend on the $BrAcT_3$ concentration, with no indication of reaching a plateau at higher concentrations of the inhibitor, thus demonstrating that the reaction can in fact be regarded as bimolecular. Furthermore, this finding also indicates that there is no specific high-affinity binding site for thyroid hormones on the Mi_b-CK molecule.

Inactivation of papain with bromoacyl compounds

Papain at a concentration of 4 μM (90 μg/ml) was incubated at 25 °C in buffer B with 10 μM bromoacetate, 40 μM phenacyl bromide or 2-(2-bromoacetyl)naphthalene, or 100 μM bromoacetamide, methyl bromoacetate or $BrAcT_3$. Tenfold-concentrated stock solutions of the bromoacyl compounds in methanol were used. After various time intervals, samples of the incubation mixtures were withdrawn and papain activity was directly determined by the pH-Stat method in an assay mixture containing 56 mM BAEE, 5 mM cysteine, 2 mM EDTA and 0.3 M NaCl. NaOH (20 mM) was used as titrant. One unit corresponds to 1 μmol of BAEE hydrolysed/min at pH 6.20 and 25 °C. The inactivation rate constants were calculated as described above.

Modification of cysteine by bromoacyl compounds

Cysteine at a concentration of 1 mM (when modified by bromoacetate or bromoacetamide) or 0.1 mM [methyl bromoacetate, phenacyl bromide, 2-(2-bromoacetyl)naphthalene, $BrAcT_3$] was incubated at 25 °C in buffer B devoid of 2-ME with 2 mM bromoacetate or bromoacetamide, 0.2 mM methyl bromoacetate, phenacyl bromide or 2-(2-bromoacetyl)-naphthalene, or 0.3 mM $BrAcT_3$. Tenfold-concentrated stock solutions of the bromoacyl compounds in methanol were used. After various time intervals, samples were withdrawn, diluted to 1.2 ml with bidistilled water, and mixed with 50 μl of 20 mM DTNB, 50 mM Na_2HPO_4 and 100 μl of 0.75 M Tris, 15 mM EDTA, pH 8.2. After 5 min, the amount of thiophenolate ions, liberated by the reaction of DTNB with the thiol group of unmodified cysteine, was determined spectrophotometrically at 412 nm. The modification rate constants were calculated as described above.

Binding of thyroid hormones and derivatives to chicken CK isoenzymes

CK-binding assays were performed essentially as previously described for transthyretin [38]. Pure CK isoenzymes (final concentration 1.2–120 μg/ml) were incubated with 30000 c.p.m. of L-3,3′,5-tri[^{125}I]iodothyronine or L-3,3′,5,5′-tetra[^{125}I]iodothyronine (specific radioactivity > 1200 μCi/μg; Amersham Buchler G.m.b.H., Braunschweig, Germany) in a total volume of 100 μl of buffer C (0.1 M Tris/HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA) in the absence or presence of unlabelled thyroid hormones or thyroid hormone derivatives (1 nM–20 μM) for 10–30 min at 25 °C or 37 °C. The reaction was stopped by the addition of 500 μl of ice-cold dextran-coated charcoal (8.3 g of activated charcoal Norit A and 0.83 g of Dextran T70 suspended in 1 litre of buffer C; Sigma, Heidelberg, Germany), followed by an additional incubation of the mixture for 10 min at 4 °C. Free and bound hormones were separated by centrifugation at 3000 *g* for 10 min at 4 °C. Non-specific binding was determined by tracer displacement with saturating concentrations of T_3 or T_4 (20 μM). Purified human transthyretin (kindly provided by Dr. V. Cody, Medical Foundation, Buffalo, NY, U.S.A.) served as positive control in these binding and displacement experiments [38].

Other methods

SDS/PAGE and autoradiography were performed as described by Köhrle et al. [18]. For the immunodetection of the CK isoenzymes, SDS/polyacrylamide gels were semi-dry-blotted on to nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany) in an SDS-containing transfer buffer for 105 min at a constant current of 1 mA/cm^2 [39]. Incubation with the primary and secondary antibodies as well as peroxidase staining with chloronaphthol as substrate were carried out as described [40]. Rabbit anti-(chicken Mi_a-, Mi_b-, M- and B-CK) sera, diluted 1:250 with buffer D (10% horse serum in PBS, 1 mM NaN_3, pH 7.2–7.4) or 5% (w/v) non-fat dried milk, were used as primary antibodies, and horse-radish peroxidase-labelled goat anti-rabbit IgG (Nordic, Biogenzia Lemania AG, Lausanne, Switzerland) diluted 1:2500 with the same buffers as secondary antibody. Protein concentrations were determined by the method of Bradford [41] using the Bio-Rad reagent and, as standard, BSA or bovine γ-globulin. An f.p.l.c. Superose 12 column (Pharmacia) was used for gel-permeation chromatography to determine the dimer to octamer ratio of the Mi-CK isoenzymes [35]. Cellulose polyacetate electrophoresis was performed as described [34]. Statistical analysis was done by Student's t test, and all results are means ± S.D. for three or four measurements.

In extraction experiments, rat heart mitochondria were swollen with bidistilled water or with bidistilled water containing 10 μM T_3 or 10 μM $BrAcT_3$. After centrifugation of the mitoplasts, Mi-CK was extracted in buffer E (100 mM sodium phosphate, 10 mM 2-ME, 1 mM NaN_3, pH 7.4), buffer E plus 10 μM T_3 or buffer E plus 10 μM $BrAcT_3$. The membranes were pelleted again and the CK activities determined in the pellets and supernatants.

RESULTS

Characterization of the CK isoenzyme system of the rat

Cardiac muscle and brain from rat [42,43], chicken [34,40,44] and man [45] were previously reported to contain two different Mi-CK isoenzymes. The chicken Mi-CK isoenzymes were further shown to form dimeric and octameric molecules [34,35,40]. The two oligomeric forms migrate as separate bands in cellulose polyacetate electrophoresis experiments [34] and are readily interconvertible. For example, formation of a transition-state-analogue complex of the Mi-CK isoenzymes with MgADP, creatine, plus nitrate leads to complete dissociation of the octameric molecules into dimers with no change in enzyme

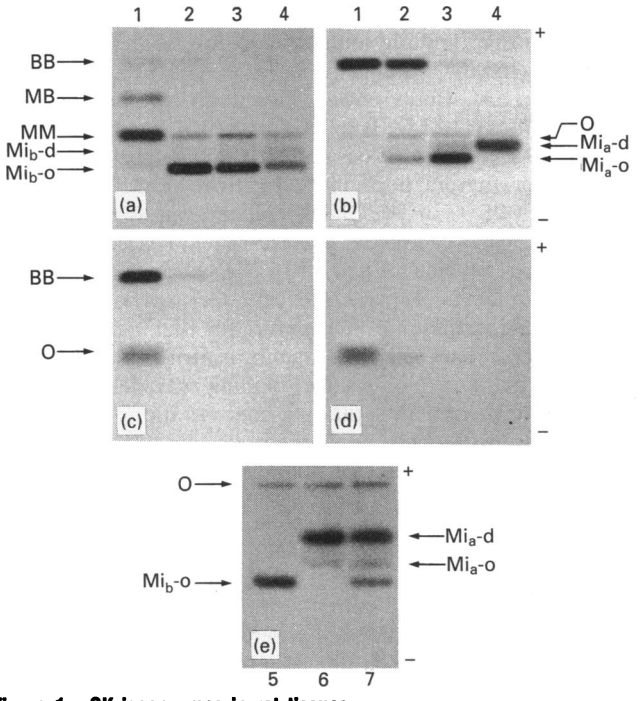

Figure 1 CK isoenzymes in rat tissues

Cellulose polyacetate electrophoresis of subcellular fractions of heart, brain and liver was performed in veronal buffer at pH 8.6, at room temperature, and at a constant voltage of 100 V for 45 (**a–d**) or 90 (**e**) min. The CK isoenzymes were then stained by a coupled enzyme assay. (**a**) Rat heart. (**b**) Rat brain. (**c**, **d**) Rat liver; (**d**) represents a negative control, since the coupled CK assay was performed in the absence of PCr. The visible band in lane 1 therefore represents adenylate kinase activity. (**e**) Comigration of the rat heart and brain Mi-CK isoenzymes. Lanes 1, cytosolic fractions; lanes 2, mitochondrial fractions; lanes 3, mitoplast fractions in the absence of substrates; lanes 4, mitoplast fractions after an 18 h incubation at 4 °C with 4 mM ADP, 5 mM $MgCl_2$, 20 mM creatine and 50 mM KNO_3 in buffer B; lane 5, heart mitochondria; lane 6, brain mitoplasts; lane 7, comigration of heart mitochondria and brain mitoplasts; O, origin; Mi$_a$-d, Mi$_a$-o, Mi$_b$-d, Mi$_b$-o, dimers and octamers of Mi$_a$- and Mi$_b$-CK respectively. Note that the staining in the region of MM-CK in the mitochondrial and mitoplast fractions of heart and brain is not due to MM-CK. Electrophoresis at a different pH (not shown) demonstrated that instead it is due to deposited material which during electrophoresis remained at the origin.

activity [34,35]. In order to confirm these findings also for the rat, and to be able to distinguish the binding of $BrAcT_3$ to each of the different CK isoenzymes, subfractionations of rat heart, brain and liver were performed, and the CK isoenzymes of the respective fractions subsequently analysed by cellulose polyacetate electrophoresis (Figure 1). Liver was included as a negative control for it is known to contain only minute amounts of CK activity [46].

As can be seen in Figure 1(a), the cytosolic and mitochondrial CK isoenzymes of rat heart were nicely separated by differential centrifugation. In contrast with chicken Mi$_a$- and Mi$_b$-CK, formation of a transition-state-analogue complex of rat heart Mi-CK was always accompanied by a progressive loss of activity, and a faint dimer band could only rarely be observed (Figure 1a, lane 4). Immunoblotting experiments confirmed that this loss of activity was not due to the formation of inactive Mi-CK dimers, but probably to denaturation of the protein (not shown).

The brain mitochondrial fraction (Figure 1b, lane 2) proved to be heavily contaminated by synaptosomal BB-CK. However, disruption of the synaptosomes by swelling in bidistilled water allowed the complete removal of the contaminating BB-CK (Figure 1b, lane 3). In contrast with the situation with rat heart Mi$_b$-CK, two separate bands for dimeric and octameric Mi$_a$-CK were observed in rat brain, and dissociation of Mi$_a$-CK octamers into dimers was achieved by dilution, high salt or urea treatment (not shown), or by formation of a transition-state-analogue complex (Figure 1b, lane 4) with no significant change in activity. Electrophoretic comigration experiments further corroborated the finding that rat heart and brain indeed contain two different Mi-CK isoenzymes (Figure 1e). Rat kidney, lung, stomach and uterus also contain the brain isoform of Mi-CK (not shown).

In contrast with the cytosolic fractions of rat heart (6.51 units/mg of protein) and brain (7.27 units/mg of protein), liver cytosol contains only minute amounts of CK activity (0.054 unit/mg of protein). This activity is entirely due to BB-CK (Figure 1c, lane 1), for the second band in the region of MM-CK turned out to represent adenylate kinase activity (Figure 1d, lane 1). These findings contrast with the detection of significant amounts of Mi-CK in human liver [47] and also conflict with results of Biermans et al. [46] who reported that rat liver contains 53% BB-, 8% MB- and 38% MM-CK. As plausible explanations, species differences and, in the latter study, misidentification of adenylate kinase as MM-CK have to be considered.

Labelling and inactivation of the CK isoenzymes by $BrAcT_3$

In order to prove that the protein doublet labelled by BrAc[^{125}I]T_3 in rat heart mitochondria [9] in fact represents Mi-CK and to decide whether this specific binding of $BrAcT_3$ is a particular property of the cardiac Mi$_b$-CK isoenzyme or a more general feature of all CK isoenzymes, the experiments shown in Figures 2 and 3 were performed. Incubation of purified chicken Mi-CK isoenzymes with BrAc[^{125}I]T_3 resulted in strong incorporation of radioactive label (Figure 2a). In the different subcellular fractions of rat heart, brain and liver, $BrAcT_3$ labelled a distinct number of proteins in various amounts. Antibody staining of electroblots (Figure 2b) confirmed that the main band labelled in rat heart mitochondria (Figure 2a, lane 4) indeed comigrates with Mi$_b$-CK. Staining with an anti-(Mi$_a$-CK) antibody (cross-reacting with all CK isoenzymes; Figure 2c) and with an anti-(B-CK) antibody (being specific for the cytosolic isoenzymes M- and B-CK; Figure 2d) further revealed that M-CK in rat heart cytosol (Figure 2, lane 3), B-CK in rat brain cytosol (lane 5) and Mi$_a$-CK in rat brain mitoplasts (lane 6) were also labelled by BrAc[^{125}I]T_3, although less selectively than Mi$_b$-CK. Almost no labelling in the 43 kDa region was observed in rat liver cytosol and mitochondria, as expected from the very low amounts of CK activity found in this tissue. The low incorporation yield into cytosolic M-CK (lane 3) is probably due to trapping of the thyroid hormone derivative by rat serum albumin (heavily labelled band in lane 3). The main band labelled in rat liver mitochondria with an M_r of about 55000 probably represents protein disulphide isomerase present in high concentrations in contaminating microsomes [16,17].

The finding that all CK isoenzymes are labelled by $BrAcT_3$ was further corroborated by inactivation experiments with the purified chicken CK isoenzymes (Figure 3 and Table 1A). Mi$_a$-CK displayed the highest inactivation rate, followed by B-, Mi$_b$- and M-CK.

At this point, two possibilities had to be considered: (i) all CK isoenzymes are targets of thyroid hormones and are potentially regulated by them; (ii) the labelling of the CK isoenzymes is simply due to the alkylation by $BrAcT_3$ of the extraordinarily reactive thiol group of CK (Cys-278, numbering according to chicken heart Mi$_b$-CK [44]) with no prior involvement of a thyroid-hormone-binding site on CK itself. The following experi-

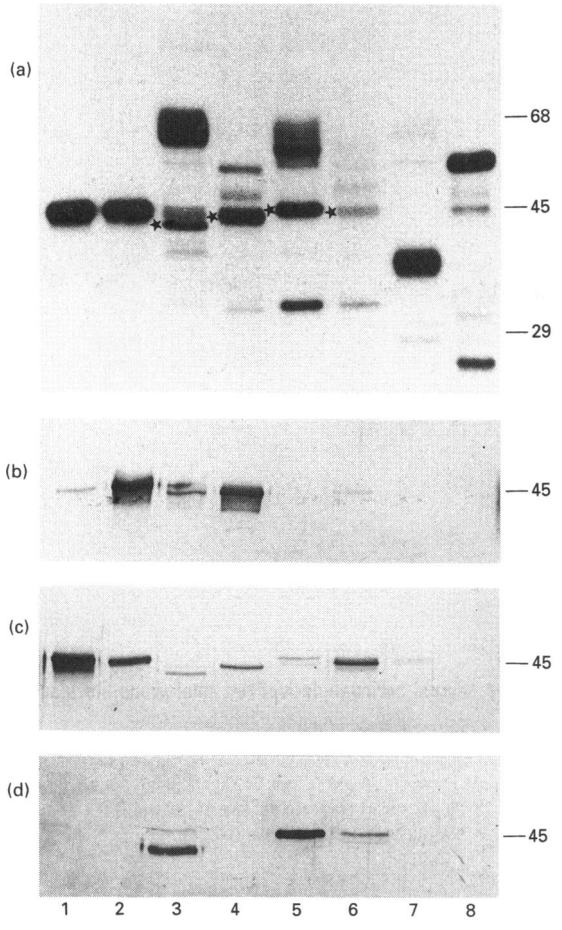

Figure 2 Labelling of the subcellular fractions of rat heart, brain and liver with BrAc[^{125}I]T_3

The labelling experiments were performed for 15 min as described in the Materials and methods section. The labelled subcellular fractions were subjected to SDS/PAGE, semi-dry-blotted, and the blots autoradiographed (**a**) and stained with antibodies against chicken Mi_b- (**b**), Mi_a- (**c**) or B-CK (**d**). Lane 1, purified chicken Mi_a-CK; lane 2, purified chicken Mi_b-CK; lane 3, rat heart cytosol; lane 4, rat heart mitochondria; lane 5, rat brain cytosol; lane 6, rat brain mitoplasts; lane 7, rat liver cytosol; lane 8, rat liver mitochondria. In (**a**), the labelled bands in the cytosolic and mitochondrial fractions of rat heart and brain corresponding to the four CK isoenzymes are marked by small stars at the left of the respective lanes; M-CK (lane 3), Mi_b-CK (lane 4), B-CK (lane 5) and Mi_a-CK (lane 6).

ments were designed to discriminate between these two possibilities.

Competition experiments

Even 20 μM concentrations of thyroid hormones and thyroid hormone derivatives such as the 5′-deiodinase inhibitors iopanoic acid and EMD 21388 (which are not metabolized) did not or only slightly reduce the radioactive labelling by $BrAcT_3$ of rat heart Mi_b-CK as well as of chicken Mi_a- and Mi_b-CK (Figure 4, Table 2). In contrast, thiol-group-blocking reagents such as 2,4-dinitrofluorobenzene (DNFB) and iodoacetamide, as well as excess concentrations of unlabelled bromoacetyl compounds led to a marked diminution of the labelling intensity of the Mi-CK band. Excess concentrations of 3,5-T_2, T_3 and T_4 (100 μM) were also not effective in preventing inactivation of chicken Mi_b-CK by $BrAcT_3$, therefore indicating that interaction of $BrAcT_3$ with

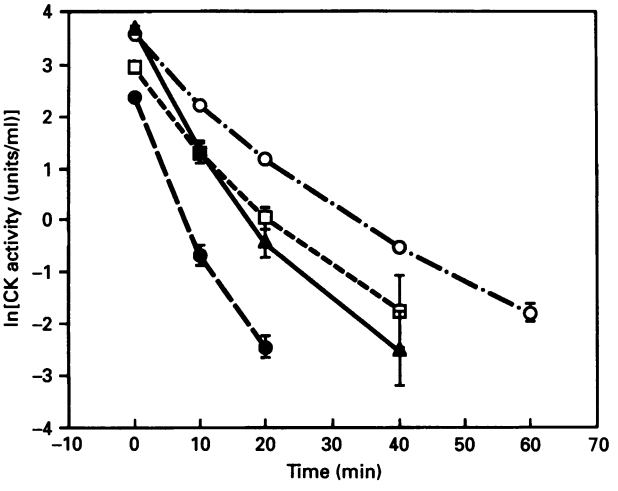

Figure 3 Inactivation of purified chicken CK isoenzymes by $BrAcT_3$

The inactivation experiments were performed in buffer B as described in detail in the Materials and methods section: 0.17 mg/ml (= 4 μM of subunits) of chicken Mi_a- (●), Mi_b- (□), M- (○) and B-CK (▲) were incubated at 25 °C with 10 μM $BrAcT_3$ for the indicated periods of time, and the CK activities thereafter determined by the pH-Stat method.

Table 1 Initial-rate constants for the inactivation of chicken CK isoenzymes by $BrAcT_3$ in the presence or absence of thyroid hormones or CK substrates

The initial inactivation rate constants were calculated from the 0 and 10 min values of inactivation experiments as described in the Materials and methods section. (A) The initial inactivation rate constants were calculated from the data presented in Figure 3. (B) The following concentrations were used: Mi_b-CK, 4 μM (0.17 mg/ml); $BrAcT_3$, 10 μM; 3,5-T_2, T_3 and T_4, 100 μM each. (C) The following concentrations were used: Mi_b-CK, 4 μM (0.17 mg/ml); $BrAcT_3$, 10 μM; ADP and ATP, 4 mM each; $MgCl_2$, 5 mM; creatine and PCr, 20 mM each; and KNO_3, 50 mM. For experiments B and C, the significance of difference relative to the control conditions is indicated.

Components	Initial inactivation rate constants ($M^{-1} \cdot s^{-1}$)	Significance (*t* test)
(A) Inactivation of the chicken CK isoenzymes by $BrAcT_3$		
Mi_a-CK	710 ± 94	
Mi_b-CK	355 ± 47	
M-CK	282 ± 8	
B-CK	526 ± 47	
(B) Effect of thyroid hormones on the inactivation of chicken Mi_b-CK by $BrAcT_3$		
None (control)	429 ± 52	
3,5-T_2	467 ± 16	
T_3	585 ± 67	$P < 0.02$
T_4	551 ± 12	$P < 0.005$
(C) Effect of substrates on the inactivation of chicken Mi_b-CK by $BrAcT_3$		
None (control)	336 ± 25	
MgADP	923 ± 71	$P < 0.001$
MgATP	490 ± 54	$P < 0.01$
Creatine	331 ± 14	
PCr	348 ± 16	
MgADP + creatine	338 ± 28	
MgATP + creatine	322 ± 30	
MgADP + creatine + nitrate	110 ± 9	$P < 0.001$

a thyroid-hormone-binding site is not a prerequisite for the covalent modification of Mi_b-CK (Table 1B). The increases in radiolabelling and inactivation rates of the chicken Mi-CK isoenzymes by $BrAcT_3$ in the presence of high concentrations of

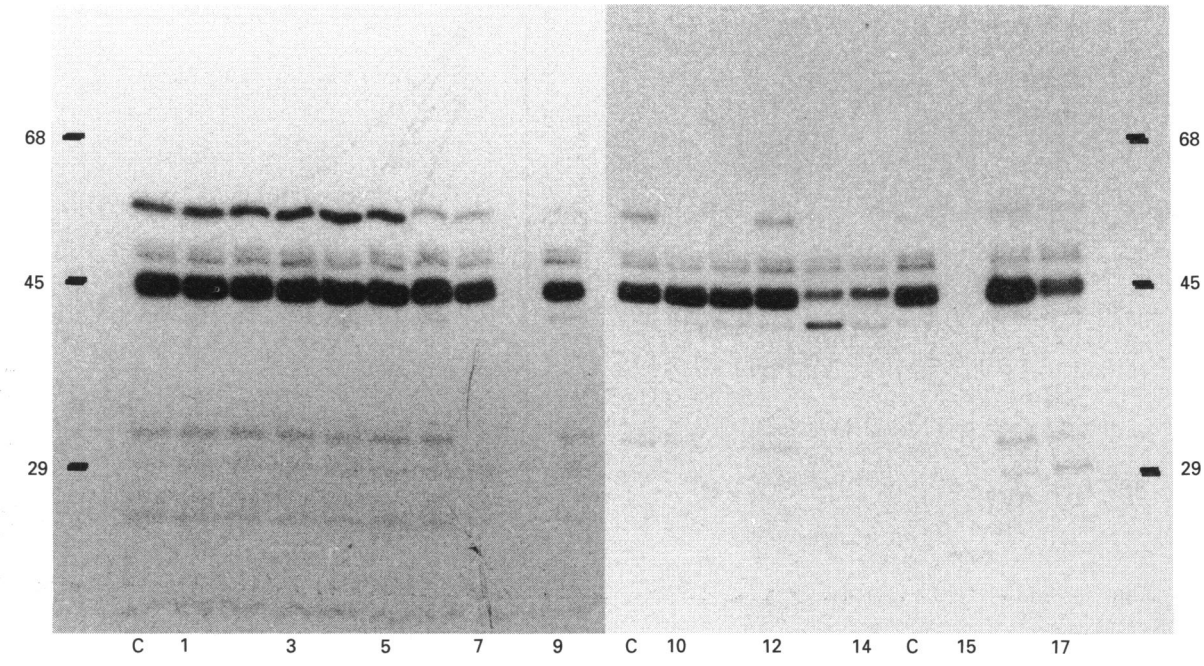

Figure 4 Competition of the $BrAcT_3$ labelling of rat heart mitochondria with thyroid hormones, thyroid hormone derivatives, thiol-group-blocking reagents or substrates

Labelling of rat heart mitochondria with $BrAc[^{125}I]T_3$ was performed for 15 min as described in the Materials and methods section in buffer A in the presence of the following competitors: C, control without competitor; lane 1, 200 nM 3,5-T_2; lane 2, 20 μM 3,5-T_2; lane 3, 200 nM T_3; lane 4, 20 μM T_3; lane 5, 200 nM T_4; lane 6, 20 μM T_4; lane 7, 200 nM $BrAcT_3$; lane 8, 20 μM $BrAcT_3$; lane 9, 20 μM EMD 21388; lane 10, 20 μM $NAcT_3$; lane 11, 20 μM $NAcT_4$; lane 12, 20 μM iopanoic acid; lane 13, 20 μM methyl bromoacetate; lane 14, 20 μM DNFB; lane 15, 10 mM iodoacetamide; lane 16, 4 mM ADP + 5 mM $MgCl_2$; lane 17, 4 mM ADP + 5 mM $MgCl_2$ + 20 mM creatine + 50 mM KNO_3 (transition-state-analogue complex).

Table 2 Competition exerted by thyroid hormones, thyroid hormone derivatives, thiol-group-blocking reagents and CK substrates on the labelling of rat heart mitochondria as well as purified chicken Mi_a- and Mi_b-CK by $BrAc[^{125}I]T_3$

Labelling of rat heart mitochondria and purified chicken Mi_a- and Mi_b-CK by $BrAc[^{125}I]T_3$ was performed in buffer A for 15 min at 25 °C as described in the Materials and methods section. Relative incorporation yields were determined by ethanol precipitation of protein-bound radioactivity. The amount of radioactivity incorporated under the appropriate conditions in the absence of competitors was taken as 100%. Five-fold-concentrated stock solutions of CK substrates and iodoacetamide in buffer A and 50-fold-concentrated stock solutions of thyroid hormones, thyroid hormone derivatives, DNFB and methyl bromoacetate in methanol were used.

		Radioactivity incorporated (%)		
Competitors	Concentration	Rat heart mitochondria	Chicken Mi_b-CK	Chicken Mi_a-CK
Control		100.0	100.0	100.0
T_2	200 nM	97.9 ± 0.3	102.1 ± 2.8	–
T_2	20 μM	103.1 ± 2.0	100.0 ± 3.4	112.7 ± 5.0
T_3	200 nM	96.3 ± 1.0	105.4 ± 5.3	–
T_3	20 μM	99.2 ± 4.2	115.7 ± 4.8	188.5 ± 12.9
T_4	200 nM	93.7 ± 1.6	98.4 ± 7.4	–
T_4	20 μM	90.4 ± 1.0	99.7 ± 5.6	195.7 ± 7.3
$NAcT_3$	20 μM	89.7 ± 3.2	80.5 ± 5.4	–
$NAcT_4$	20 μM	88.7 ± 2.5	80.0 ± 3.5	–
EMD 21388	20 μM	76.8 ± 2.2	121.5 ± 9.3	–
Iopanoic acid	20 μM	103.1 ± 7.6	114.4 ± 2.1	–
$BrAcT_3$	200 nM	71.0 ± 1.7	64.3 ± 8.4	51.3 ± 1.0
$BrAcT_3$	20 μM	22.4 ± 1.4	13.8 ± 3.9	8.2 ± 1.3
Methyl bromoacetate	20 μM	77.4 ± 1.7	41.8 ± 3.1	–
DNFB	20 μM	67.9 ± 4.4	31.3 ± 4.8	–
Iodoacetamide	10 mM	16.8 ± 2.3	14.9 ± 1.7	12.9 ± 2.5
MgADP		171.7 ± 3.6	102.5 ± 1.8	148.8 ± 0.7
Transition-state-analogue complex		112.3 ± 10.6	52.4 ± 1.8	44.9 ± 17.1

thyroid hormones may be explained by reduced non-specific binding of $BrAcT_3$ to Mi-CK or mitochondrial membranes and/or by partial protection of $BrAcT_3$ from degradation [9].

The facts that MgADP enhances the inactivation rate of chicken Mi_b-CK and that formation of a transition-state-analogue complex protects the enzyme from inactivation (Table 1C) perfectly agree with experiments on rabbit MM-CK in which inhibition of the enzyme activity by iodoacetamide was also increased by MgADP and diminished by formation of a transition-state-analogue complex [48]. Taken together, these results show that $BrAcT_3$, as well as thiol-group-blocking reagents that have a low structural similarity to thyroid hormones, behave similarly towards CK, indicating that the selective labelling of the CK isoenzymes by $BrAcT_3$ is triggered by their highly reactive thiol groups and not by iodothyronine-binding sites.

Binding of underivatized T_3 to chicken Mi_b-CK

No specific binding of $[^{125}I]T_3$ or $[^{125}I]T_4$ and also no displacement of non-specifically bound thyroid hormones (4–8 % of total) by unlabelled T_3, T_4 or thyroid hormone derivatives was observed for purified chicken Mi_a- and Mi_b-CK under experimental conditions which allowed detection of specific thyroid-hormone binding and ligand competition for transthyretin. These results are in accordance with the lack of effects of thyroid hormones and thyroid hormone derivatives in competition studies of the CK inactivation and labelling experiments and also indicate the absence of a specific thyroid-hormone-binding site in CK isoenzymes.

Inactivation of chicken Mi_b-CK by other bromoacyl compounds

In order to corroborate the conclusions drawn so far and to see if bromoacyl compounds in general might be selective labelling and inactivation reagents for CK isoenzymes, the interaction of six bromoacyl compounds with Mi_b-CK was investigated. For comparison, the rates of modification of papain, a cysteine proteinase, as well as of cysteine itself were also determined for the same reagents. Phenacyl bromide and 2-(2-bromoacetyl)-naphthalene reacted very rapidly with available thiol groups (Figure 5, Table 3). In the presence of 0.5 mM 2-ME, inactivation

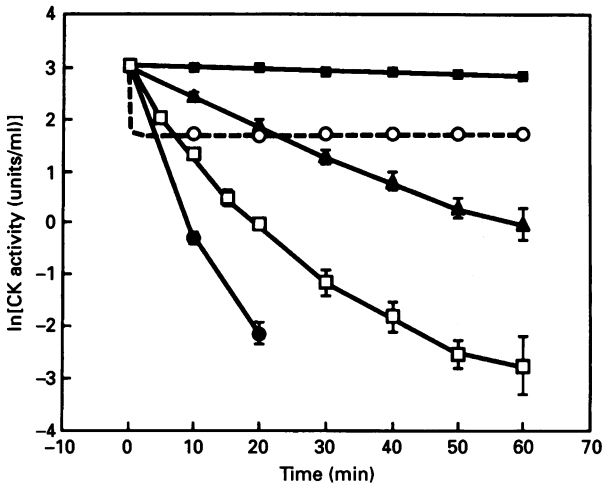

Figure 5 Inactivation of chicken Mi_b-CK by different bromoacyl compounds

The inactivation experiments were performed in buffer B as described in the Materials and methods section. The following concentrations were used: Mi_b-CK, 4 μM (0.17 mg/ml); bromoacetic acid and 2-bromoacetamide, 100 μM each; $BrAcT_3$, methyl bromoacetate; phenacyl bromide, 10 μM each. ■, Bromoacetic acid; ▲, 2-bromoacetamide; □, $BrAcT_3$; ●, methyl bromoacetate; ○, phenacyl bromide. When Mi_b-CK was incubated under the same conditions, but without a bromoacyl compound present, no significant decrease in enzyme activity was observed after 1 h.

of Mi_b-CK was not complete. In the absence of 2-ME, however, complete inactivation by phenacyl bromide and 2-(2-bromoacetyl)naphthalene was already achieved after 10 min (not shown). In contrast with these very electrophilic reagents, the other bromoacyl compounds tested reacted preferentially with Mi_b-CK, even in the presence of 0.5 mM 2-ME, as can be seen from the almost straight lines in the semilogarithmic plot (Figure 5). When, on a structural basis, $BrAcT_3$ is assumed to have about the same inherent reactivity as 2-bromoacetamide, its reaction rate with Mi_b-CK is increased 40-fold by the thyroid hormone moiety (Table 3). When, however, the rate of cysteine modification is taken as a direct measure of the reactivity of the bromoacetyl compounds, the reaction rate of $BrAcT_3$ with Mi_b-CK, relative to 2-bromoacetamide, is augmented by 300-fold. A

Table 3 Reaction rate constants for the inactivation of Mi_b-CK and papain as well as for the modification of cysteine by different bromoacyl compounds

The reaction rate constants were calculated as described in the Materials and methods section. The values for the inactivation of Mi_b-CK are derived from the data presented in Figure 5. 2-(2-Bromoacetyl)naphthalene was used at a concentration of 10 μM. The experiments on the inactivation of papain and on the modification of cysteine by different bromoacyl compounds were performed as described in the Materials and methods section.

	Reaction rate constants ($M^{-1} \cdot s^{-1}$)		
Bromoacyl compound	Inactivation of Mi_b-CK	Inactivation of papain	Modification of cysteine
Bromoacetic acid	0.368 ± 0.522	318.3 ± 28.3	0.115 ± 0.025
2-Bromoacetamide	9.72 ± 1.39	41.1 ± 2.9	0.698 ± 0.029
$BrAcT_3$	388 ± 32	2.32 ± 0.28	0.090 ± 0.020
Methyl bromoacetate	785 ± 33	8.00 ± 0.48	5.64 ± 0.46
Phenacyl bromide	Very fast	Faster than BAA*	†
2-(2-Bromoacetyl)naphthalene	Very fast	Faster than BAA*	†

* The rate of inactivation of papain on incubation with phenacyl bromide or 2-(2-bromoacetyl)naphthalene was higher than with bromoacetic acid (BAA).

† No information can be given on the rate constants for the modification of cysteine by phenacyl bromide and 2-(2-bromoacetyl)naphthalene. After an initial increase in A_{412} during the titration with DTNB, the yellow colour rapidly disappeared. The reason for this effect might be the modification of the thiophenolate ion, liberated during the titration reaction, by the two highly reactive bromoacyl compounds.

40- to 300-fold increase corresponds to a difference in activation energy of approximately 9–14 kJ/mol which is not sufficient to postulate a specific thyroid-hormone-binding site of Mi_b-CK, but supports the notion that the unusually reactive thiol group of CK is located within a hydrophobic environment [32,49].

To conclude, bromoacetyl compounds are very promising candidates for the specific labelling and inactivation of CK isoenzymes. Because of the hydrophobic pocket near the reactive thiol group of CK, an aromatic moiety attached to the bromoacetyl group is likely to increase the specificity of the reagent, as can be seen from the data obtained with $BrAcT_3$. However, the bromoacyl moiety should not be part of a delocalized system as in phenacyl bromide or 2-(2-bromoacetyl)-naphthalene, since these reagents are too reactive and thus bind non-specifically to all available thiol groups.

DISCUSSION

In the present investigation, Mi-CK was identified as a target for alkylation by the thyroid hormone derivative $BrAcT_3$, whereas under the same conditions the mitochondrial adenine nucleotide translocator (ANT) was not labelled in rat heart, brain or liver. However, a large body of evidence demonstrates that Mi-CK, like ANT, is in all likelihood not a mitochondrial thyroid-hormone-binding protein. (1) 3,5-T_2, T_3, T_4 and thyroid hormone analogues such as EMD 21388 were not effective in protecting rat heart Mi_b-CK as well as purified chicken Mi_a- and Mi_b-CK from labelling by $BrAc[^{125}I]T_3$ (Figure 4, Table 2). In addition, they did also not decrease the rate of inactivation of chicken Mi_b-CK by $BrAcT_3$ (Table 1B). (2) No specific binding of underivatized T_3 to purified chicken Mi_b-CK was observed. (3) Extraction experiments with rat heart mitoplasts manifested no influence of 10 μM T_3 on the interaction of Mi_b-CK with the inner mitochondrial membrane. Furthermore, 20 μM T_3 or 3,5-T_2 did not affect the enzyme activity and only slightly increased the dimer to octamer ratio of both chicken Mi_a- and Mi_b-CK (results not shown). (4) MgADP on the one hand and formation of a transition-state-analogue complex on the other influenced the labelling and inactivation of Mi_b-CK by $BrAcT_3$ in the very same way (Figure 4, Table 1C) as they influenced the inactivation of rabbit MM-CK by iodoacetamide [48], indicating that either a reactive thiol group is close to or directly involved in a thyroid-hormone-binding site of Mi_b-CK or that the interaction of $BrAcT_3$ with Mi_b-CK is entirely due to the extraordinary reactivity of some thiol groups of CK (probably Cys-278). The findings that pure thiol-group-blocking reagents such as iodoacetamide and DNFB were most effective in competing for the labelling of Mi_b-CK by $BrAc[^{125}I]T_3$ (Figure 4, Table 2), that all CK isoenzymes in rat and chicken were modified by $BrAcT_3$ (Figures 2 and 3), with no marked preference for the mitochondrial isoenzymes, and that other bromoacyl compounds with no structural similarity to thyroid hormones also inactivated chicken Mi_b-CK in the same concentration range as $BrAcT_3$ (Figure 5, Table 3) strongly favour the second possibility.

These findings open the door to the design of very specific inhibitors and labelling reagents for CK, i.e. by combining the substrate specificity of the CK isoenzymes with the extraordinary selectivity of bromoacetyl derivatives for the highly reactive thiol group of CK. Since the latter was reported to be located at or near the substrate-binding site of CK, and since ATP γ-*p*-azidoanilide [50] and C1R-ATP [51] proved to be valuable reagents for the identification of amino acid residues near the active site of CK, ADP or ATP analogues with a bromoacetyl moiety attached to the β- or γ-phosphate group respectively will be promising candidates for future studies.

As far as the thyroid hormone aspect is concerned, the present study also provides some important implications. Thyroid hormones have a variety of acute as well as long-term effects on mitochondria. They are known to increase mitochondrial oxygen consumption and oxidative phosphorylation rate [52,53], mitochondrial DNA, RNA and protein synthesis ([54]; for reviews see [6–8]), mitochondrial proton leakage and membrane potential [55], as well as the extramitochondrial ATP/ADP ratio [55,56], whereas they decrease the intramitochondrial ATP/ADP ratio [53,56]. Furthermore, thyroid hormones influence mitochondrial morphology and turnover [57] and are thought to regulate the enzyme activities of several mitochondrial enzymes (for a review see [58]). Whether they also decrease the ADP/O and H^+/O ratios is still disputed (see [58–60]).

Some effects of thyroid hormones on mitochondria may be accounted for by regulation of nuclear gene expression, but others are suggested to be direct effects, since they were also observed in isolated mitochondria or in the presence of specific inhibitors of cytoplasmic protein synthesis [7,52,53,59]. In fact, several groups have demonstrated the presence of saturable high-affinity T_3-binding sites in mitochondria from a variety of tissues ([23,24,61–63]; for a review see [7]) which were localized both to the inner [7,23,24,63] and outer [63] membranes. Purification of a thyroid-hormone-binding protein from mitochondrial inner membranes [61], its physicochemical similarity to ANT, and photoaffinity-labelling by T_3 of purified ANT from bovine heart mitochondria, a preparation that displayed high-affinity low-capacity T_3-binding sites, led to the hypothesis that ANT is the actual 'mitochondrial thyroid hormone receptor' [62]. Several arguments, however, favour the contrary [6,55,64]. Most importantly, the reported binding capacity of the purified mitochondrial T_3 'receptor' (143 fmol/mg of protein) [61] is far below the value expected for a 1:1 ratio of thyroid hormone to ANT (32 nmol/mg of protein on the basis of a subunit M_r of 31000 for the ANT) meaning that a faint contamination in the ANT preparation might be responsible for the specific T_3-binding activity.

In accordance with these conclusions, Rasmussen et al. [9] were not able to label ANT in rat heart mitochondria with $BrAcT_3$. Instead, a protein doublet of equal intensity of M_r about 45000 was preferentially labelled. Since the labelling of this protein doublet is simply due to the extraordinary reactivity of the bromoacetyl moiety of $BrAcT_3$ with the highly reactive thiol group of Mi-CK, the question of how thyroid hormone effects on mitochondria are mediated still remains unsolved. Several possibilities have to be taken into account. (1) Mitochondria may have a different thyroid hormone specificity than the nucleus where T_3 displayed the highest affinity for the c-*erbA*-related receptor proteins [3]. Since 3,5-T_2 in perfused rat liver has been shown to increase oxygen consumption and oxidative phosphorylation [52,53], BrAc-3,5-T_2 may be an attractive alternative for future studies. (2) Thyroid hormones may exert their effects on mitochondria by altering the fluidity of the inner mitochondrial membrane, either directly by insertion into the lipid bilayer or indirectly by regulation of lipid metabolism [65]. (3) Some groups did not observe any thyroid hormone effects on isolated mitochondria, unless they injected T_3 or T_4 into severely hypothyroid animals several hours before isolation of the mitochondria [66–68]. Therefore the hypothesis was raised that cytoplasmic factors mediate the thyroid hormone effects on mitochondria [67–69].

The following additional inferences can be drawn from the present investigation. Apart from a 43000–47000-M_r band which very probably represents a fragment of the nuclear T_3 receptor [12,70], some proteins in the M_r 45000 range that were modified

by $BrAcT_3$ or by photoaffinity-labelling with underivatized T_3 [10,11,14,20,70,71] may represent CK isoenzymes. The most attractive candidate is the 43000-M_r polypeptide labelled by $BrAcT_3$ in a human placental plasma-membrane fraction [14] which may actually be Mi-CK, for (a) placenta is known to contain Mi_a-CK [45], (b) the native and subunit M_r values of the placental protein determined by gel filtration and SDS/PAGE seem to agree with the expected values for octameric and monomeric Mi-CK, (c) mitochondrial contamination of the plasma-membrane fraction used by Alderson et al. [14] was reduced by only a factor of 4.5, and (d) labelling of the placental 43000-M_r protein by $BrAcT_3$ was also not reduced by excess amounts of T_3.

Whereas Siegel et al. [72] and Cahnmann et al. [21] postulated that specificity problems with $BrAcT_3$ or $BrAcT_4$ [10,12–14,17,20,72] are due to the fact that bromoacetyl derivatives of thyroid hormones act as exo- rather than endo-affinity labels, the present study favours the hypothesis that the lack of specificity for thyroid-hormone-binding sites is due to the preferential reaction of these derivatives with particular thiol groups. Interestingly, incubation of bovine heart mitochondria with bromodaunomycin, a bromoacetyl derivative of adriamycin, led to the selective labelling of three proteins, one of which had a subunit M_r of 45000 [73] which is again indicative of Mi-CK.

Professor H. M. Eppenberger, Professor R. D. Hesch and Professor P. W. Jungblut are gratefully acknowledged for continuous support and critical reading of the manuscript, E. Zanolla for expert technical assistance, Professor H. Dutler, Dr. M. Oertel and Dr. E. Furter-Graves for valuable discussions, and Dr. H. Rokos, Dr. K. Irmscher and Dr. V. Cody for kindly providing chemical reagents. This work was supported by grants from the Swiss National Science Foundation (31-26384.89), the Swiss Society for Muscle Diseases and the Deutsche Forschungsgemeinschaft (He 593/21-1).

REFERENCES

1 Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. and Eppenberger, H. M. (1992) Biochem. J. **281**, 21–40
2 Wyss, M., Smeitink, J., Wevers, R. A. and Wallimann, T. (1992) Biochim. Biophys. Acta **1102**, 119–166
3 Oppenheimer, J. H., Schwartz, H. L., Mariash, C. N., Kinlaw, W. B., Wong, N. C. W. and Freake, H. C. (1987) Endocrine Rev. **8**, 288–308
4 Segal, J. (1990) Endocrinology **127**, 17–24
5 Siegrist-Kaiser, C. A., Juge-Aubry, C., Tranter, M. P., Ekenbarger, D. M. and Leonard, J. L. (1990) J. Biol. Chem. **265**, 5296–5302
6 Höppner, W., Horst, C., Rasmussen, U. B. and Seitz, H. J. (1987) Horm. Metab. Res. Suppl. **17**, 29–33
7 Sterling, K. (1989) Endocrinol. Res. **15**, 683–715
8 Nelson, B. D. (1990) Biochim. Biophys. Acta **1018**, 275–277
9 Rasmussen, U. B., Köhrle, J., Rokos, H. and Hesch, R.-D. (1989) FEBS Lett. **255**, 385–390
10 Horiuchi, R., Johnson, M. L., Willingham, M. C., Pastan, I. and Cheng, S.-Y. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 5527–5531
11 van der Walt, B., Nikodem, V. M. and Cahnmann, H. J. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 3508–3512
12 Pascual, A., Casanova, J. and Samuels, H. H. (1982) J. Biol. Chem. **257**, 9640–9647
13 Cheng, S.-Y. (1983) Endocrinology **113**, 1155–1157
14 Alderson, R., Pastan, I. and Cheng, S.-Y. (1985) Endocrinology **116**, 2621–2630
15 Hasumura, S., Kitagawa, S., Lovelace, E., Willingham, M. C., Pastan, I. and Cheng, S. (1986) Biochemistry **25**, 7881–7888
16 Yamauchi, K., Yamamoto, T., Hayashi, H., Koya, S., Takikawa, H., Toyoshima, K. and Horiuchi, R. (1987) Biochem. Biophys. Res. Commun. **146**, 1485–1492
17 Schoenmakers, C. H. H., Pigmans, I. G. A. J., Hawkins, H. C., Freedman, R. B. and Visser, T. J. (1989) Biochem. Biophys. Res. Commun. **162**, 857–868
18 Köhrle, J., Rasmussen, U. B., Rokos, H., Leonard, J. L. and Hesch, R. D. (1990) J. Biol. Chem. **265**, 6146–6154
19 Köhrle, J., Rasmussen, U. B., Ekenbarger, D. M., Alex, S., Rokos, H., Hesch, R. D. and Leonard, J. L. (1990) J. Biol. Chem. **265**, 6155–6163
20 Gonçalves, E., Lakshmanan, M., Cahnmann, H. J. and Robbins, J. (1990) Biochim. Biophys. Acta **1055**, 151–156
21 Cahnmann, H. J., Gonçalves, E., Ito, Y., Fales, H. M. and Sokoloski, E. A. (1992) Anal. Biochem. **204**, 344–350
22 Cheneval, D. (1987) Dissertation no. 8377, ETH Zürich, Switzerland
23 Sterling, K., Milch, P. O., Brenner, M. A. and Lazarus, J. H. (1977) Science **197**, 996–999
24 Goglia, F., Torresani, J., Bugli, P., Barletta, A. and Liverini, G. (1981) Pflügers Arch. Eur. J. Physiol. **390**, 120–124
25 Schlegel, J., Wyss, M., Eppenberger, H. M. and Wallimann, T. (1990) J. Biol. Chem. **265**, 9221–9227
26 Kupriyanov, V. V., Steinschneider, A. Ya., Ruuge, E. K., Kapel'ko, V. I., Zueva, M. Yu., Lakomkin, V. L., Smirnov, V. N. and Saks, V. A. (1984) Biochim. Biophys. Acta **805**, 319–331
27 Jacobus, W. E. (1985) Annu. Rev. Physiol. **47**, 707–725
28 Taurog, A. (1986) in Werner's The Thyroid (Ingbar, S. H. and Braverman, L. E., eds.), 5th edn., pp. 53–97, J. B. Lippincott Co., Philadelphia
29 Rao, M. L. and Rao, G. S. (1982) Biochem. J. **206**, 19–25
30 Köhrle, J., Hesch, R. D. and Leonard, J. L. (1991) in Werner's and Ingbar's The Thyroid (Braverman, L. E. and Utiger, R. D., eds.), 6th edn., pp. 144–189, J. B. Lippincott Co., Philadelphia
31 Menezes Ferreira, M. M., Bismuth, J. and Torresani, J. (1982) Biochem. Biophys. Res. Commun. **105**, 244–251
32 Watts, D. C. (1973) in The Enzymes (Boyer, P. D., ed.), 3rd edn., Vol. 8, pp. 383–455, Academic Press, New York
33 Kuby, S. A. and Noltmann, E. A. (1962) in The Enzymes (Boyer, P. D., Lardy, H. and Myrbäck, K., eds.), 2nd edn., Vol. 6, pp. 515–603, Academic Press, New York
34 Wyss, M., Schlegel, J., James, P., Eppenberger, H. M. and Wallimann, T. (1990) J. Biol. Chem. **265**, 15900–15908
35 Schlegel, J., Zurbriggen, B., Wegmann, G., Wyss, M., Eppenberger, H. M. and Wallimann, T. (1988) J. Biol. Chem. **263**, 16942–16953
36 Quest, A. F. G., Eppenberger, H. M. and Wallimann, T. (1989) Enzyme **41**, 33–42
37 Eppenberger, H. M., Dawson, D. M. and Kaplan, N. O. (1967) J. Biol. Chem. **242**, 204–209 and 210–217
38 Köhrle, J., Auf'mkolk, M., Rokos, H., Hesch, R.-D. and Cody, V. (1986) J. Biol. Chem. **261**, 11613–11622
39 Kyhse-Andersen, J. (1984) J. Biophys. Biochem. Methods **10**, 203–209
40 Schlegel, J., Wyss, M., Schürch, U., Schnyder, T., Quest, A., Wegmann, G., Eppenberger, H. M. and Wallimann, T. (1988) J. Biol. Chem. **263**, 16963–16969
41 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
42 Sanders, J. L., Joung, J. I. and Rochman, H. (1976) Biochim. Biophys. Acta **438**, 407–411
43 Payne, R. M., Haas, R. C. and Strauss, A. W. (1991) Biochim. Biophys. Acta **1089**, 352–361
44 Hossle, J. P., Schlegel, J., Wegmann, G., Wyss, M., Böhlen, P., Eppenberger, H. M., Wallimann, T. and Perriard, J.-C. (1988) Biochem. Biophys. Res. Commun. **151**, 408–416
45 Haas, R. C. and Strauss, A. W. (1990) J. Biol. Chem. **265**, 6921–6927
46 Biermans, W., Bakker, A. and Jacob, W. (1990) Biochim. Biophys. Acta **1018**, 225–228
47 Kanemitsu, F., Kawanishi, I. and Mizushima, J. (1982) Clin. Chim. Acta **119**, 307–317
48 Milner-White, E. J. and Kelly, I. D. (1976) Biochem. J. **157**, 23–31
49 Haugland, R. P. (1975) J. Supramol. Struct. **3**, 192–199
50 Vandest, P., Labbe, J.-P. and Kassab, R. (1980) Eur. J. Biochem. **104**, 433–442
51 James, P., Wyss, M., Lutsenko, S., Wallimann, T. and Carafoli, E. (1990) FEBS Lett. **273**, 139–143
52 Horst, C., Rokos, H. and Seitz, H. J. (1989) Biochem. J. **261**, 945–950
53 Horst, C., Hummerich, H., Soboll, S. and Seitz, H. J. (1989) in Hormones, Thermogenesis, and Obesity (Lardy, H. and Stratman, F., eds.), pp. 311–323, Elsevier Science Publishing Co., Amsterdam
54 Goglia, F., Liverini, G., Lanni, A. and Barletta, A. (1988) Mol. Cell. Endocrinol. **55**, 141–147
55 Verhoeven, A. J., Kamer, P., Groen, A. K. and Tager, J. M. (1985) Biochem. J. **226**, 183–192
56 Seitz, H. J., Müller, M. J. and Soboll, S. (1985) Biochem. J. **227**, 149–153
57 Jakovcic, S., Swift, H. H., Gross, N. J. and Rabinowitz, M. (1978) J. Cell Biol. **77**, 887–901
58 Brand, M. D. and Murphy, M. P. (1987) Biol. Rev. **62**, 141–193
59 Thomas, W. E. and Mowbray, J. (1987) Biochem. Soc. Trans. **15**, 669–670
60 Hafner, R. P. and Brand, M. D. (1988) Biochem. J. **250**, 477–484
61 Sterling, K., Campbell, G. A. and Brenner, M. A. (1984) Acta Endocrinol. (Copenhagen) **105**, 391–397
62 Sterling, K. (1986) Endocrinology **119**, 292–295
63 Hashizume, K. and Ichikawa, K. (1982) Biochem. Biophys. Res. Commun. **106**, 920–926

64 Rasmussen, U. B. and Wohlrab, H. (1986) Biochem. Biophys. Res. Commun. **138**, 850–857
65 Hoch, F. L. (1992) Biochim. Biophys. Acta **1113**, 71–133
66 Nelson, B. D., Joste, V., Wielburski, A. and Rosenqvist, U. (1980) Biochim. Biophys. Acta **608**, 422–426
67 Palacios-Romero, R. and Mowbray, J. (1979) Biochem. J. **184**, 527–538
68 Higuti, T. and Rottenberg, H. (1986) Chem. Pharm. Bull. **34**, 4331–4334
69 Ichikawa, K., Hashizume, K., Kobayashi, M. and Yamada, T. (1985) Endocrinology **117**, 1749–1758
70 David-Inouye, Y., Somack, R., Nordeen, S. K., Apriletti, J. W., Baxter, J. D. and Eberhardt, N. L. (1982) Endocrinology (Baltimore) **111**, 1758–1760
71 Dozin, B., Cahnmann, H. J. and Nikodem, V. M. (1985) Biochemistry **24**, 5203–5208
72 Siegel, J. S., Korcek, L. and Tabachnick, M. (1983) Endocrinology **113**, 2173–2180
73 Cheneval, D., Müller, M. and Carafoli, E. (1983) FEBS Lett. **159**, 123–126

Received 7 September 1992/3 November 1992; accepted 9 November 1992